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Aquaporin expression in breast cancer and their involvement in bleb formation, cell motility and invasion in endocrine resistant variant cells

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Abstract. Estrogen receptor (ER)-silenced breast cancer cell lines exhibit endocrine resistance and morphological changes from an epithelial to a mesenchymal phenotype. These cells also display increased motility and invasive properties that are further accentuated by exposure to an alkaline pH, exhibiting dynamic plasma membrane blebbing and cytoplasmic streaming. These latter morphological changes are hypothesized to involve substantial water movement across the plasma membrane, contributing to bleb formation; this may involve aquaporin channel proteins (AQPs). AQP 1, 3, 4 and 5 expression/localization was examined via reverse transcription-quantitative PCR, western blotting and confocal microscopy in endocrine-sensitive (YS1.2) and -resistant (pII and MDA-MB-231) breast cancer cells, as well as normal breast epithelial cells (MCF10A). The effects of osmotic changes on bleb formation were examined via live cell imaging. AQP3 protein expression was knocked down by small interfering RNA (siRNA) transfection, and the effect of its reduced expression on bleb formation, cell motility and invasion were determined via immunofluorescence, scratch and Cultrex assays, respectively. Expression of the four AQPs varied across the different cell lines, and exhibited nuclear, cytoplasmic and membranous localization. Osmotic changes affected the formation of blebs. In pII cells exposed to alkaline pH, AQP3 was observed to be redistributed from the nucleus into the newly formed blebs. siRNA-mediated knockdown of AQP3 in pII cells significantly reduced cellular blebbing induced by alkaline pH, as well as motility and invasion. These data suggested that AQP3, and potentially other aquaporins, may participate in the processes leading to blebbing of

endocrine-resistant cells which is proposed to be a mechanism that drives tumor metastasis.

Introduction

Breast cancer is the most commonly diagnosed neoplasm and the leading cause of cancer mortality among females worldwide (1,2). Clinically, it is divided into three main subtypes based on hormone receptor expression (3-6). One of the main characteristics of aggressive breast cancers is epithelial-mesenchymal transition (EMT), via which cells transition from an epithelial to a mesenchymal phenotype, a phenomenon that has been closely linked to metastasis, drug resistance and cancer recurrence, and reported to result in poor prognosis (7). Hallmarks of EMT include the downregulation of E-cadherin, decreased expression of claudins and occludins at tight junctions, and repression of genes encoding desmoplakin and plakophilin desmosomes (8). Reductions in the levels of these epithelial proteins disrupt cell junction complexes and cytoskeletal connections (9); occurring in parallel with activation of genes such as N-cadherin, vimentin, fibronectin, α-smooth muscle actin and matrix metalloproteinases (MMPs), this gives cells a mesenchymal-like phenotype (10,11). EMT increases cancer cell motility and facilitates invasive behavior by enabling cells to penetrate through the extracellular matrix, and invade lymphatic and vascular elements (7).

In the process of studying therapeutic resistance to endocrine therapy, several cell lines have been established in our laboratory via short hairpin RNA (shRNA)-mediated knockdown of the estrogen receptor (ER) in parental endocrine-sensitive MCF-7 breast cancer cells, resulting in cells resistant to both tamoxifen and estrogen, and associated with EMT, leading to increased motility and invasion (8,11,12). One of the main characteristics of these endocrine-resistant cells, but not of endocrine-sensitive breast cancer cells or normal breast epithelial cells, is the induction of a remarkable alteration in their morphology, including cell rounding and the formation of dynamic actin-rich blebs along the outer membrane upon brief exposure to alkaline but not acidic pH conditions (13,14). Under such conditions, ER-silenced pII cells exhibit enhanced motility and migration, in part through increased MMP activity (12). These blebs can be observed at the leading

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edge of cells moving towards a chemoattractant source in an amoeboid-like manner (13-15). Bleb formation can be reversed or prevented by inhibitors of cytoplasmic streaming or drugs known to inhibit certain ion channels; the most effective of these is ouabain, a potent inhibitor of Na^+/K^+ ATPase (13,14). The reversibility of bleb formation - which disappear upon returning the cells to pH 7.4 - suggests that this may be a physiological process to promote cellular motility in order to enable cells to move away from an essentially hostile environment. The process of cytoplasmic streaming required for bleb formation must involve considerable water movement and thus is likely to be subject to osmotic changes; therefore, the expression of membrane channel proteins responsible for water transport, such as aquaporins (AQPs), is of major research interest.

AQPs are small, hydrophobic, integral transmembrane proteins whose major function is to facilitate the transport of water molecules (16,17). To date, ~300 distinct AQPs have been discovered in diverse organisms, with 13 mammalian isoforms having been identified (AQP0-AQP12). Based on their functional and structural properties, AQPs are classified into subfamilies: The classical AQPs, including AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8, are selective for water transport, whereas the aquaglyceroporins and the super-aquaporins, which include AQP3, AQP7, AQP9 and AQP10, also transport small solutes such as ammonia, urea and glycerol. AQP3 is selective for water and glycerol (16,17).

In addition to the various physiological roles of AQPs, they have also been linked to cancer pathogenesis in various organs including the lung (18), colon (19), brain (22) and liver (21). Shi *et al* (16) reported the expression of several AQPs in breast cancer, including AQPs 1, 3, 4 and 5; all were upregulated in cancer tissue apart from AQP4, which was more intensely expressed in normal tissue. AQP1 expression was detected in basal-like (22) and luminal cancer subtypes (23), and associated with poor prognosis. AQP1 overexpression was shown to enhance breast cancer proliferation and invasion *in vitro* (23), while small interfering RNA (siRNA)-mediated knockdown of AQP1 decreased tumor mass and volume *in vivo* (24). Enhanced AQP3 expression in patients with early breast cancer was associated with poor prognosis (25). Treating ER-positive (ER^+) breast cancer cells with estrogen upregulated expression of AQP3; this is likely via activation of estrogen response elements (EREs) in its gene promoter (26). Additionally, siRNA knockdown of AQP3 significantly reduced cell migration and invasion (26-28). AQP4 has been reported to be elevated in breast cancer tissues (16), and its siRNA-mediated downregulation in ER^+ breast cancer cells enhanced the expression of E-cadherin, and decreased cell proliferation, motility and invasion (29). Enhanced expression of AQP5 in tumors has been associated with poor clinical prognosis and lymph node metastasis (30), and shRNA-knockdown of AQP5 in MCF-7 cells significantly decreased cell proliferation and migration (31).

In the present study, it was determined that alkaline pH induces bleb formation in endocrine-resistant pII breast cancer cells, regulated in part by water movement in response to osmotic changes in the extracellular environment. AQP3 (and to a certain extent, AQP1) was translocated to newly formed blebs. In addition, siRNA-mediated knockdown of AQP3 in pII cells significantly decreased bleb formation, and cell motility and invasion. These data suggested that AQP3 may play a role in bleb formation/stabilization, and thereby help promote invasion and metastasis.

Materials and methods

Cell lines. The ER-negative (ER^-) MDA-MB-231 human breast carcinoma cell line was originally obtained from the American Type Culture Collection (ATCC). MCF10A normal breast epithelial cells were obtained from Dr Elizabeth Saunderson through Dr Jenny Gomm (St Bartholomew's Hospital, London). pII (ER^-) cells were generated via shRNA-mediated knockdown of ER in MCF7 cells (which were also originally obtained from the ATCC); YS1.2 was derived from similarly transfected MCF7 cells, in which ER was not downregulated; therefore, this cell line is used as ER^+ control for pII (8,11). For routine culture, all cell lines were maintained as monolayers at 37°C in an incubator with an atmosphere of 5% CO_2 at 95% humidity, in advanced DMEM containing phenol red as a pH indicator and supplemented with 5% FBS, 600 μg/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 6 ml/500 ml 100X non-essential amino acids (all from Invitrogen; Thermo Fisher Scientific, Inc.).

Morphology of pII cells exposed to alkaline pH and osmotic changes. Cells were removed from the CO_2 incubator and exposed to standard atmospheric conditions, which caused the medium pH to rise to 8.3 (13) within 10-30 min (depending on whether grown in culture plates or flasks). The morphology of cells under control (pH 7.4) and alkaline (pH 8.3) conditions was monitored via phase contrast light microscopy. Images were captured for the same field at 0 and 30 min (4 fields for each condition; magnification, x40). The number of blebbing cells was counted manually to compare the percentage of cells blebbing. Live cell microscopy with time-lapse photography was used to continuously monitor the effect of osmotic changes on the bleb morphology of pII cells. For this, cells were grown in a 10-mm culture dish containing 2 ml DMEM and exposed to standard atmospheric conditions for 30 min to alkalinize the medium. The cells were then placed inside an imaging chamber (Cell Observer HS; Carl Zeiss AG) heated with an airstream to 37°C with normal atmosphere and monitored for 120 min. Cells were photographed at x40 magnification and images were captured at 5-min intervals. This experiment was repeated by first inducing alkaline conditions for 30 min, followed by the removal of the original media, and substitution with media diluted with water (1:1 and 1:4) or sucrose solution (1-100 mM) for 30 min to produce acute hypotonic or hypertonic conditions, respectively.

Reverse transcription-quantitative PCR (RT-qPCR). RNA was extracted from cell pellets using an RNeasy kit from Qiagen, Inc., and 2 μg (in 20 μl reaction mix) was converted to cDNA using a High Capacity cDNA Reverse Transcriptase kit from Applied Biosystems (Thermo Fisher Scientific, Inc.). qPCR was performed on 1 μl of the cDNA reaction mix using a standard multiplexed TaqMan PCR kit protocol (Applied Biosystems; Thermo Fisher Scientific, Inc.) to determine the expression of AQPs. Control gene probes (human β-actin; cat. no. 4310881E;

Thermo Fisher Scientific, Inc.) were labeled with VIC™, and target gene probes for AQP1 (cat. no. Hs01028916_m1), AQP3 (cat. no. Hs00185020_m1), AQP4 (cat. no. Hs00242342_m1) and AQP5 (cat. no. Hs00387048_m1; all Applied Biosystems; Thermo Fisher Scientific, Inc.) were labeled with fluorescein amidite. The amplifications were performed on an Applied Biosystems 7500 HT Fast thermocycler under the following conditions: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The raw quantification cycle (Cq) values were converted via the ΔΔCq method to determine normalized expression ratios of the target genes relative to β-actin (32).

Western blotting. Cells were cultured in 6-well plates with complete DMEM to 80-90% confluence. Medium was subsequently aspirated off, and cell monolayers were harvested by scraping and resuspended into 300 μl of lysis buffer containing 50 mM HEPES, 50 mM NaCl, 5 mM EDTA 1% Triton X, 100 μg/ml PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Protein concentration was determined via the Bradford assay using BSA (Sigma-Aldrich; Merck KGaA) as standard, and 8 μg protein lysate was mixed with an equal volume of 2X SDS and heated at 90˚C for 10 min. Samples were loaded onto a 10% SDS-polyacrylamide gel and electrophoresed at 150 V for 1 h. Proteins were transferred to a PVDF membrane and blocked with 2% BSA for 1 h at 4˚C before being incubated overnight at 4˚C with AQP3 antibody (1:500; cat. no. ab125219; Abcam) prepared in 2% BSA. The membrane was washed and incubated with anti-HRP-conjugated secondary antibody (1:500; cat. no. 7074; Cell Signaling Technology, Inc.) for 1 h at 4˚C, developed with Super Signal ECL (Thermo Fisher Scientific, Inc.) and visualized with Kodak X-ray film. Blots were also probed with β-actin antibody (1:1,000; cat. no. 8457; Cell Signaling Technology, Inc.) as a loading control.

Immunofluorescence analysis. Cells were seeded at ~5,000 cells/well in an 8-well chambered slide containing 300 μl/well DMEM, and left to grow under standard culture conditions for 2 days. pII cells were also cultured under standard conditions for 2 days before subsequent exposure to alkaline pH conditions for 30 min. Media were removed, and cells were immediately fixed by addition of 3.7% formaldehyde (200 μl/well) for 10 min with gentle agitation at room temperature. Alternatively, cells were fixed by incubation with ice-cold 100% methanol for 15 min at -20˚C. Removal of the fixative was followed by three washing steps with ice-cold PBS, each for 5 min. Non-specific binding sites were blocked by addition of 1% BSA in PBS (100 μl), and cells were incubated at room temperature for 1 h. The BSA was then aspirated, and 200 μl/well of primary antibody solution was added and left to incubate overnight at 4˚C, with anti-AQP1 (cat. no. ab11025), anti-AQP3 (cat. no. ab125219), anti-AQP4 (cat. no. ab46182) or anti-AQP5 (cat. no. ab15119) antibodies added (all 1:100; all Abcam). Following removal of the primary antibodies, cells were washed as aforementioned and the secondary antibody (Alexa Fluor® 555-conjugated anti-rabbit IgG; 1:500; cat. no. 4413; Cell Signaling Technology, Inc.) was added for a further 2-h incubation at room temperature in the dark. This was followed by a repeat of the previously described washing steps after removal of the antibody solution.

Then, 200 μl/well of diluted phalloidin (6 μl in 1 ml PBS; Thermo Fisher Scientific, Inc.) was added and incubated for 10 min at room temperature in the dark. After removing the phalloidin and washing with PBS three times, the chambers and borders of the 8-well chambered slides were removed, and a drop of DAPI was added onto the slide immediately prior to mounting. Staining was visualized and photographed using an LSM 510 Meta confocal microscope (Carl Zeiss AG) at an excitation wavelength of 450 nm.

For comparison, experiments were also performed using AQP antibodies sourced from Biorbyt Ltd. [AQP1 (cat. no. orb10122), AQP3 (cat. no. orb47955), AQP4 (cat. no. orb10125) and AQP5 (cat. no. orb395791); all 1:100] and Santa Cruz Biotechnology, Inc. [AQP1 (cat. no. sc-25287), AQP3 (cat. no. sc-518001), AQP4 (cat. no. sc-32739) and AQP5 (cat. no. sc-514022); all 1:100; data not shown].

AQP3 siRNA transfection. Cells were plated in 12-well plates in complete DMEM, incubated for 24 h at 37˚C with 5% CO_2, and allowed to grow until reaching 70-90% confluence. Transfection was then performed using control scramble siRNA (cat. no. sc-37007) or AQP3 siRNA (cat. no. sc-29713; both Santa Cruz Biotechnology, Inc.). Solution A was prepared by adding 6 μl Lipofectamine® RNAiMAX transfection reagent (cat. no. 56532; Invitrogen; Thermo Fisher Scientific, Inc.) to 100 μl of Opti-MEM reduced serum medium (Invitrogen; Thermo Fisher Scientific, Inc.), and solution B was prepared by diluting 2 μl of AQP3 siRNA into 100 μl of Opti-MEM reduced serum medium (0.2 μM final concentration). Solutions A and B were mixed, incubated for 15 min and added dropwise to the cells. Cells were then incubated at 37˚C with 5% CO_2. Following 48-72-h incubation, cells were harvested and RNA was extracted for the determination of AQP3 expression via RT-qPCR. Other transfected cells were harvested at 72 h for AQP3 protein determination via western blotting, or after 48 h for motility and invasion assays, as well as determining the effects of alkaline pH on cell morphology via immunofluorescence confocal microscopy.

Motility assay. Cells (control and following AQP3 siRNA transfection for 48 h) were cultured in 12-well plates with complete DMEM containing 5% FBS to 80-90% confluence. A scratch was created in the cell monolayer using a sterile P1000 pipette tip, and a photograph of the scratched area was captured immediately (0 h). After overnight incubation, another photograph (both magnification, x10) was captured of the same scratched area. The width of the scratch at 24 h was calculated as a percentage of the width at 0 h; a minimum of three areas along the scratch were measured.

Cultrex basement membrane extract (BME) cell invasion assay. Cell invasion was assessed using a Cultrex 24-well BME cell invasion assay purchased from Trevigen, Inc. (cat. no. 3455-024-K) according to the manufacturer's instructions. For this, the invasion chamber was coated with 100 μl of 1X BME solution and incubated overnight at 37˚C. After 24 h of transfection, cells were serum-starved overnight at 37˚C with 5% CO_2. On the following day (after 48 h of transfection), pII cells were harvested, counted and diluted to $1x10^6$ cells/ml in serum-free medium. After that, 100 μl of cells were added
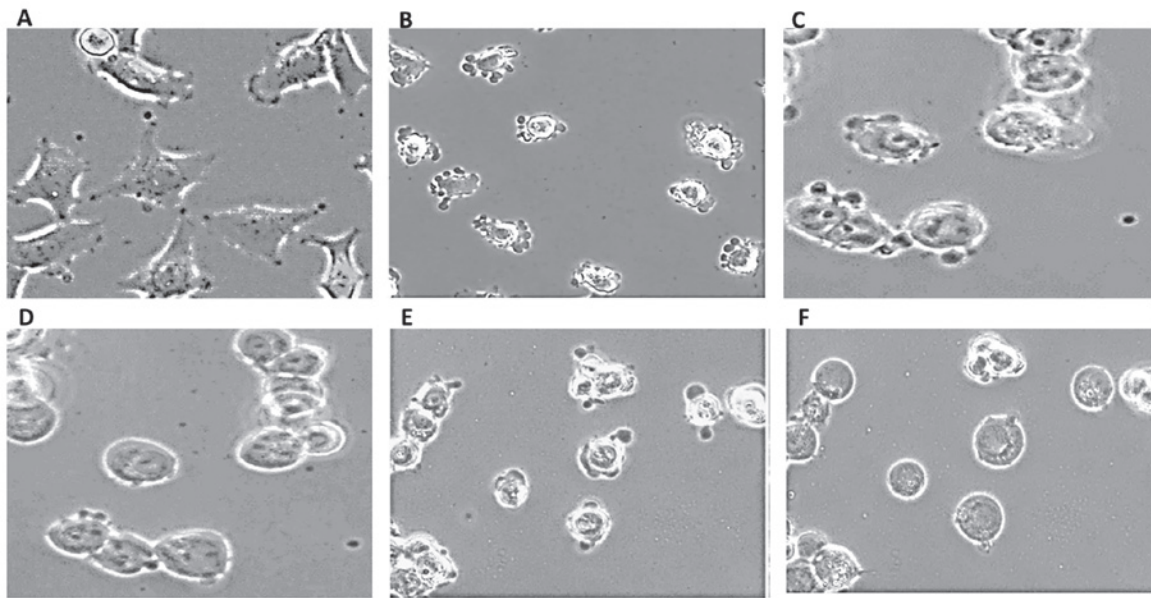
Figure 1. Effect of hypotonic conditions on alkaline pH-induced bleb formation in pII cells. Representative images of pII cells (grown in 10-mm culture dishes in DMEM at 37°C and 5% CO_2) taken by phase contrast light microscopy (A) immediately after removal from incubator at pH 7.4 or (B) after exposure to alkaline conditions (pH 8.3) for 30 min. In a separate experimental setup, cells were first exposed to alkaline pH for 30 min until they formed blebs, and the media was then removed and replaced with a solution composed of (C) DMEM diluted with water (1:1) for 30 min, (D) diluted 1:1 for 60 min, (E) diluted 1:4 for 30 min or (F) diluted 1:4 for 60 min. Cells were observed at x40 magnification in a live cell imager heated to 37°C with an airstream under normal atmospheric conditions.

to the top chamber of the Cultrex dish. The lower chamber was loaded with 500 μl of DMEM supplemented with 30% FBS (used as a chemoattractant). Cells were incubated at 37°C with 5% CO_2 and allowed to invade to the bottom chamber. After 24 h, the top and the bottom chambers were aspirated and washed with 1X cell wash buffer. Calcein-AM/cell dissociation solution complex was added to the bottom chamber and left for 1 h at 37°C with 5% CO_2. Cells internalize calcein-acetomethylester (AM), and intracellular esterases cleave the AM moiety, generating fluorescence-free calcein. Invading cells were determined by recording the fluorescence emission using a microplate reader, with an excitation/emission filter set of 485/535 nm.

Statistical analysis. Data are presented as the mean ± SEM of three independent experiments, and were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc.). Student's two-tailed unpaired t-test or one-way ANOVA followed by Bonferroni post hoc test were used to compare means of individual groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of alkaline pH and hypotonic/hypertonic conditions in the extracellular environment on the morphology and bleb formation of pII cells. Changes in the morphology of pII cells were recorded by photographing immediately after removal from the incubator at pH 7.4 (control; Fig. 1A), and then again after 30 min exposure to atmospheric conditions (pH 8.3; Fig. 1B). From their typical mesenchymal-like spindle morphology, the cells became rounded and exhibited extensive blebbing on the plasma membrane. To determine the effect

of osmotic changes on pH-induced bleb formation, pII cells were first exposed to alkaline conditions for 30 min (to form the blebs) followed by the removal of the original media and substitution with media diluted with water to produce acute hypotonic conditions. As shown in Fig. 1C-F, substitution with DMEM diluted 1:1 or 1:4 with water for 30-60 min caused the cells to become more rounded and enlarged in size, and after 60 min (Fig. 1D and F), the blebs had almost entirely disappeared. Notably, the cells were able to withstand severe hypotonic conditions without lysing even after exposure for 120 min (data not shown). Prior hypotonic conditions caused the cells to swell; this prevented bleb formation when the medium pH was allowed to alkalinize (data not shown).

In another experimental setup, pII cells were first exposed to alkaline pH conditions (Fig. 2A) followed by the addition of sucrose solution to the media at various concentrations (1, 10 and 100 mM) for 30 min (Fig. 2B-D, respectively) to induce acute hypertonic conditions. The cells became rounded and the blebs almost entirely disappeared. These data suggested that water movement across the plasma membrane is essential for alkaline pH-induced bleb formation in pII cells.

Expression profile of various AQPs in breast epithelial and cancer cell lines. RT-qPCR analysis showed that AQP1 was expressed at very low levels in MCF10A and non-invasive YS1.2 cells (12), but its levels were significantly higher in the ER⁻ breast cancer cells (Fig. 3A). AQP3 was highly expressed in YS1.2, followed by pII and MDA-MB-231, with the lowest expression in MCF10A cells (Fig. 3B). AQP4 was mainly expressed in MCF10A, with very low expression in breast cancer cells (Fig. 3C). Similar to AQP1 and AQP3, AQP5 was highly expressed in breast cancer cells compared with MCF10A (Fig. 3D). Western blotting was used to confirm the
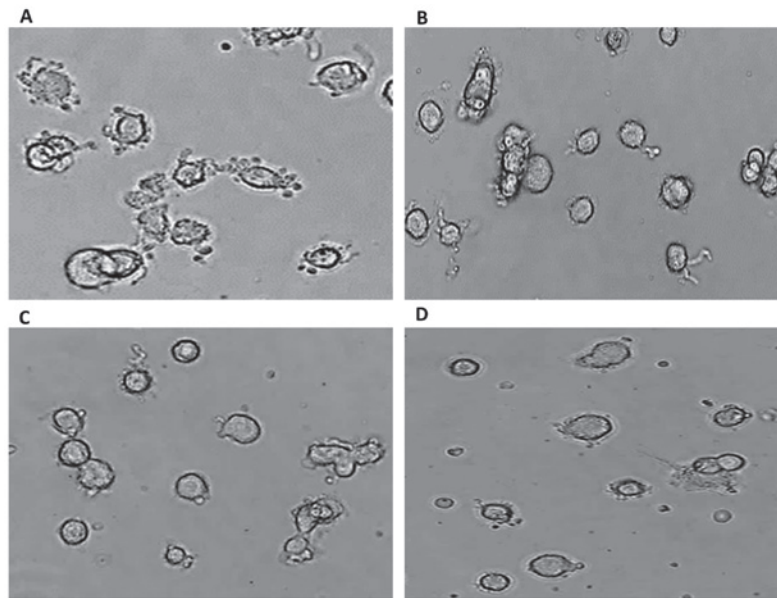
Figure 2. Effect of hypertonic conditions on alkaline pH-induced bleb formation in pII cells. (A) Representative image of pII cells (grown in 10-mm culture dishes in DMEM at 37˚C and 5% CO_2) taken by phase contrast light microscopy after exposure to alkaline conditions for 30 min. In another experimental setup, cells were first exposed to alkaline pH for 30 min until they formed blebs, and the media was then removed and replaced with a solution composed of (B) 1 mM, (C) 10 mM or (D) 100 mM sucrose for 60 min. Cells were observed at x40 magnification in a live cell imager heated to 37˚C with an airstream under normal atmospheric conditions.
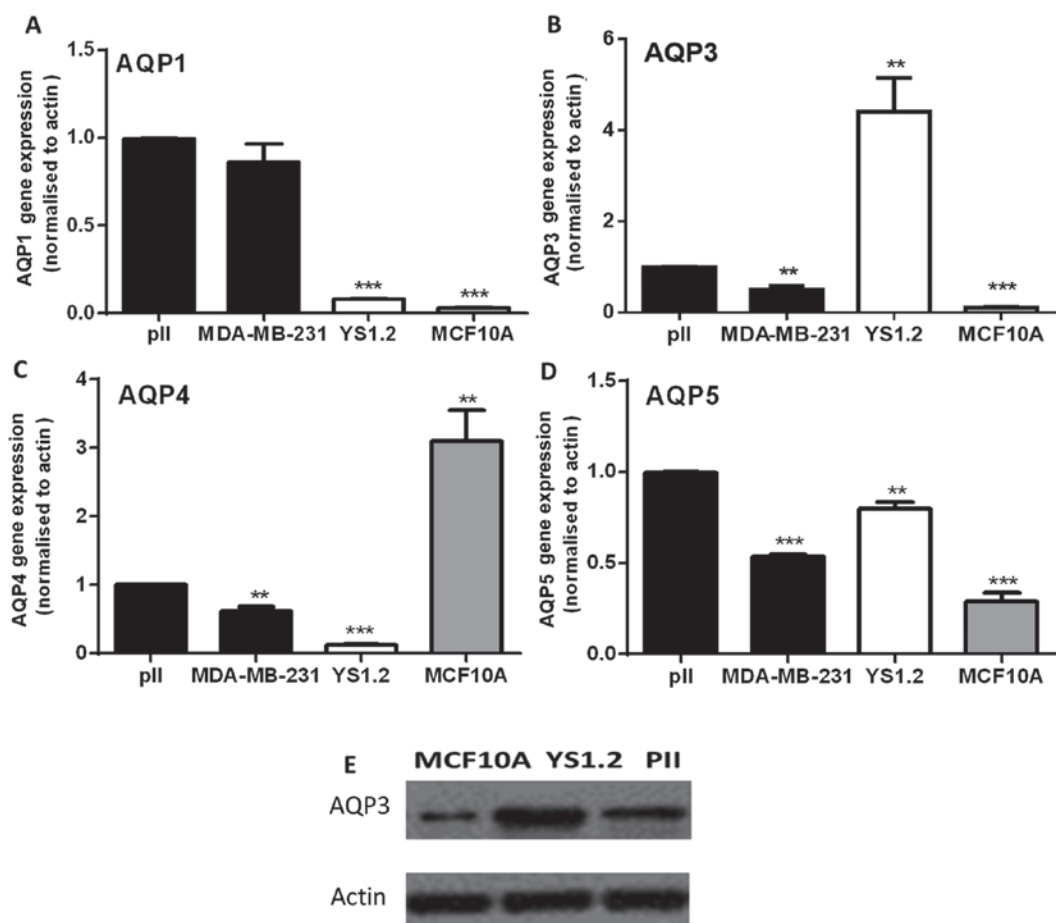

Figure 3. Expression of AQPs in normal breast MCF10A cells and breast cancer cell lines. RNA extracted from cell pellets and converted to cDNA was analyzed via reverse transcription-quantitative PCR to determine the expression of (A) AQP1, (B) AQP3, (C) AQP4 and (D) AQP4 (normalized to actin). The expression for each cell line was calculated based on quantification cycle values as a normalized ratio to the expression level observed in pII cells. (E) Western blot analysis of AQP3 protein expression. Cell lysates were separated via 10% SDS-PAGE, blotted onto PVDF membranes and probed with antibodies targeted against AQP3 and β-actin; the presented blot is representative of 3 independent experiments. Data are presented as the mean ± SEM of 3 independent experiments. **P<0.01, ***P<0.001 vs. pII. AQP, aquaporin.
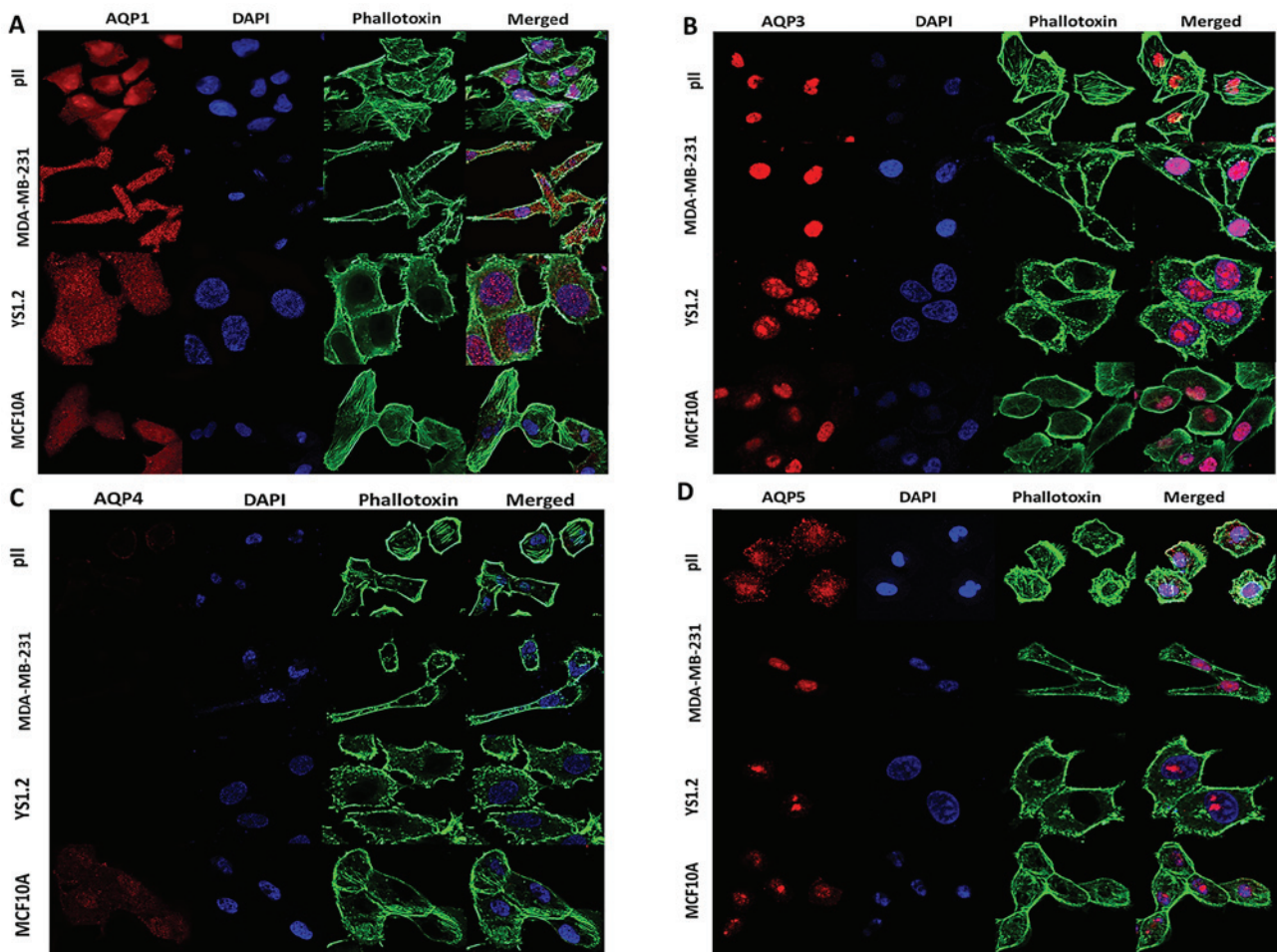
Figure 4. Immunolocalization of various AQPs in breast cell lines. Cells were grown in 10-mm culture dishes in DMEM at 37˚C and 5% CO_2 at pH 7.4 (control) and stained with (A) AQP1, (B) AQP3, (C) AQP4 and (D) AQP5 antibodies (red), as well as phalloidin (green) and DAPI (blue), before being imaged under a confocal microscope. Magnification, x60. AQP, aquaporin.

expression profile of AQP3 at the protein level in MCF10A, YS1.2 and pII cells (Fig. 3E). These results are consistent with the gene expression profile, which shows differing expression between ER⁻ and ER⁺ breast cancer cell lines. In general, AQP3 expression was higher in cancerous compared to non-cancerous breast cells. The gene expression profile of the tested AQPs was not modified in pII cells upon exposure to alkaline pH for 30 min (data not shown).

Localization pattern of various AQPs at normal pH and upon exposure to alkaline pH. The localization patterns of AQPs 1, 3, 4 and 5 in the tested cell lines cultured in pH 7.4 were determined via immunofluorescence. Due to differences in microscope settings and digital enhancement of certain images (necessary to visualise low expression), fluorescence intensities presented in the figures do not accurately reflect relative quantitative differences. AQP1 showed diffuse cytoplasmic staining in all the cell lines examined, as well as expression over the nuclear surface; immunoreactivity was weakest in MCF10A cells, confirming comparatively low levels of expression of this protein in normal breast epithelial cells (Fig. 4A). Staining with an anti-AQP3 antibody from Abcam, expression was detected only in the nuclear region, with no clear staining in the cytoplasm (Fig. 4B); this pattern was observed in all

the cell lines. In view of this somewhat unexpected distribution of AQP3 compared with other literature reports (28,33), these experiments were repeated in pII cells with antibodies purchased from other sources, which showed similar nuclear localization (data not shown). Additionally, an alternative fixation method using methanol instead of formaldehyde was used to determine whether this affected the interaction of the antibody, and similar distribution patterns were also observed (data not shown). Weak diffuse staining of AQP4 was observed only in MCF10A (Fig. 4C). AQP5 appeared to be mainly nuclear in all the cell lines, with some additional cytoplasmic immunoreactivity detected in pII cells (Fig. 4D).

The locations of AQPs 1, 3, 4 and 5 were also determined in pII cells following exposure to alkaline pH for 30 min. As presented in Fig. 5A, AQP1 exhibited a similar diffuse distribution in alkaline pH, but was now also detected inside the blebs. AQP3 remained predominantly nuclear but had also markedly migrated into the blebs, in a far more pronounced manner than AQP1 (Fig. 5B). AQP4 was not detectable at either pH (Fig. 5C). AQP5 maintained its nuclear localization, and was not observed in either the extranuclear space or inside the blebs (Fig. 5D). These data suggested that AQP3 may play an important role in pII cell migration and invasion due to its substantial translocation into the newly formed blebs.
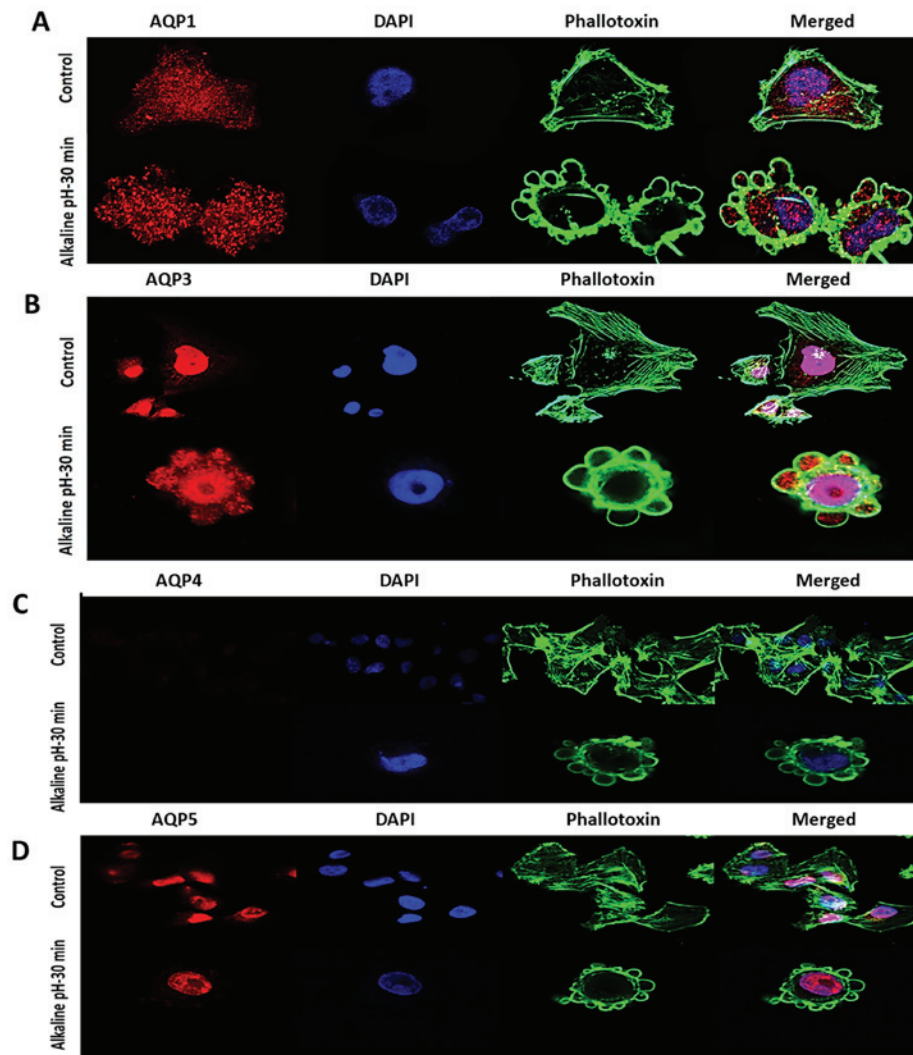
Figure 5. Effect of exposure to alkaline pH on the localization of AQPs in pII cells. pII cells were cultured in 10-mm dishes in DMEM at 37°C and 5% CO_2 at pH 7.4 (control), or exposed to pH 8.3 for 30 min, and subsequently stained with (A) AQP1, (B) AQP3, (C) AQP4 and (D) AQP5 antibodies (red), as well as phalloidin (green stain) and DAPI (blue stain), before being imaged under a confocal microscope. Magnification, x60. AQP, aquaporin.

Effects of siRNA knockdown of AQP3 on pII cell blebbing, motility and invasion. AQP3 gene expression was significantly reduced in pII cells at 48 and 72 h post-transfection with AQP3-targeting siRNA (Fig. 6A). This was paralleled by a reduction in AQP3 protein detected via western blotting at 72 h (Fig. 6B). Cultures assayed at 48 h post-transfection exhibited significant reduction in cell motility (Fig. 6C and D) and invasion towards serum components (Fig. 6E). It should be noted that the motility assays were performed in media supplemented with 5% FBS over 24 h. This is expected to result in doubling of the cell number; however, the contribution of cell proliferation to total wound closure is expected to be relatively minor. This can be clearly demonstrated by the difference between migratory and non-migratory cells cultured in growth media. Assessment of the proportion of blebbing cells in siRNA-transfected cultures exposed to alkaline pH also showed a significant reduction (Fig. 6F). The immunofluorescence images presented in Fig. 6 G show examples of the heterogeneity of response in individual cells; the left panel shows AQP3 expression in control (scrambled siRNA)-transfected blebbing cells, whereas the right panels show AQP3 siRNA-transfected cells that either failed to bleb, or blebbed despite the absence of AQP3.

Discussion

The present study revealed distinct expression profiles for AQPs 1, 3, 4 and 5 in normal epithelial breast cells and breast cancer cells. mRNA expression levels of AQPs 1, 3 and 5 were higher in breast cancer cells compared with MCF10A. Conversely, AQP4 expression was higher in MCF10A than in the cancer cells. These results are in agreement with a number of reports which demonstrated enhanced AQP 1, 3 and 5 expression in breast cancer tissues (16,22,31), particularly in those with invasive ductal carcinoma and lymph node invasion (for AQP5) (31). AQP1 expression was found to be correlated with high tumor grade and triple negativity, and exhibited a negative relationship with ER status (22). This inverse correlation is in agreement with the present findings into AQP1 mRNA expression. In contrast, the expression of AQP3 was elevated in ER$^+$ breast cancer tissues obtained from premenopausal women in comparison to those from postmenopausal
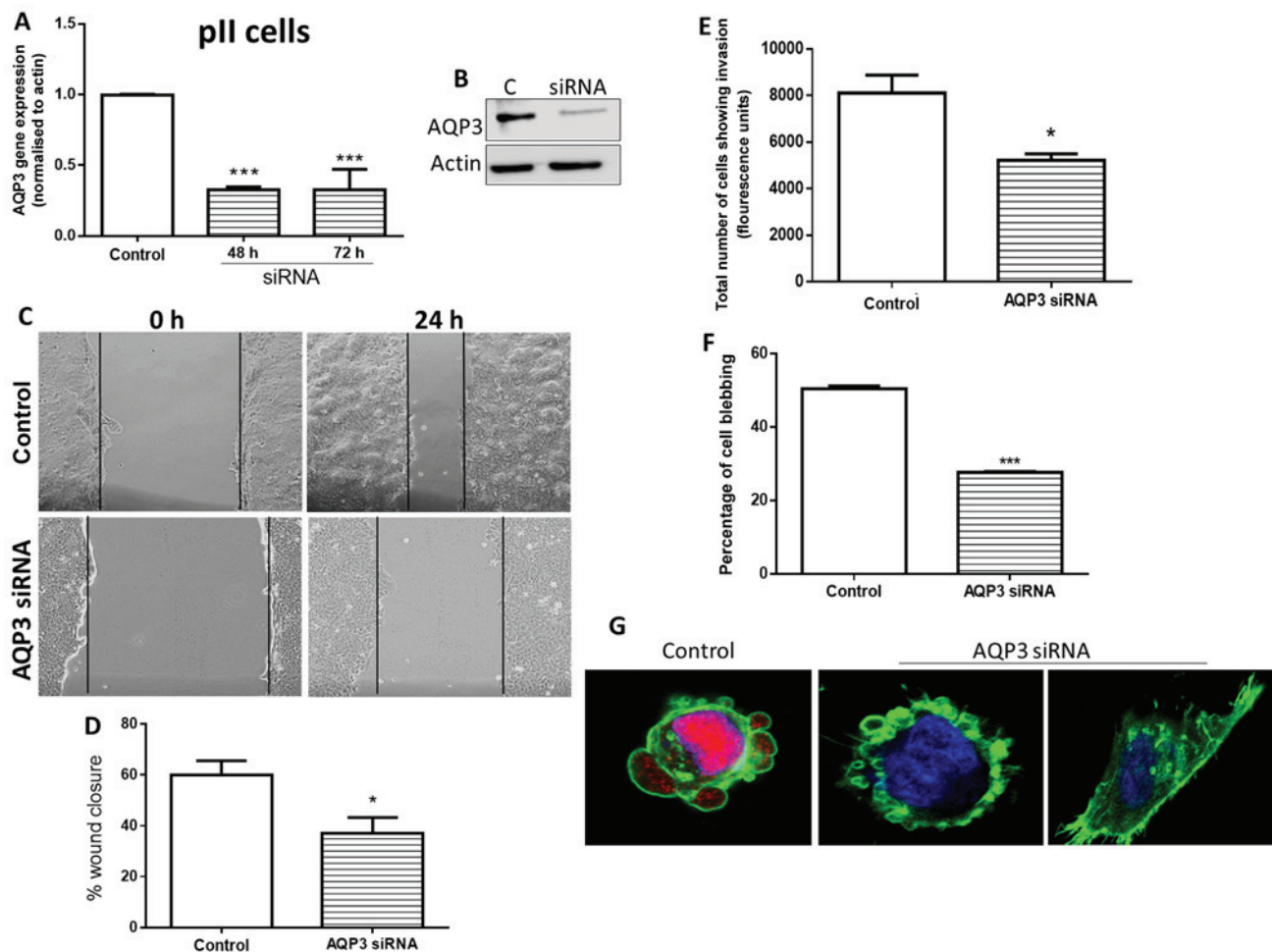
Figure 6. Effects of siRNA-mediated knockdown of AQP3 in pII cells on cell motility, invasion and alkaline pH-induced bleb formation. (A) Reverse transcription-quantitative PCR analysis of AQP3 mRNA expression in control siRNA-transfected cells, and AQP3 siRNA-transfected cells at 48 and 72 h. (B) AQP3 protein expression was determined in control siRNA- and AQP3 siRNA-transfected cells at 72 h via western blot analysis. (C) At 48 h following transfection, cells were grown to confluency, and a scratch was made through the center of the cell monolayer using a 1000 μl Eppendorf pipette. The scratch width was measured under a microscope at 0 and 24 h. (D) Percentage of wound closure was quantified. (E) Effect of AQP3 siRNA knockdown on invasion of pII cells through basement membrane extract towards serum components. The y-axis represents arbitrary fluorescence units showing uptake of calcein into the invading cells in the bottom chamber of the Cultrex dish. (F) At 48 h after transfection with AQP3 siRNA, cells were removed from the incubator and exposed to atmospheric conditions. Cells were photographed immediately (pH 7.4, control) and after 30 min (pH 8.3). The number of blebbing cells was counted manually to compare the percentage of cells blebbing between transfected and untreated pII cells. (G) Confocal images of AQP3/phalloidin/DAPI staining of individual cells in control siRNA- and AQP3 siRNA-transfected cultures exposed to alkaline pH (magnification, x60). Data are presented as the mean ± SEM of 3 independent experiments. *P<0.05, ***P<0.001 vs. control. AQP, aquaporin; siRNA, small interfering RNA.

women (33). Additionally, treating ER$^+$ (T47D and MCF7) cells and ER$^-$ (MDA-MB-231) cells with estrogen enhanced AQP3 mRNA levels only in the ER$^+$ cells (26), which was attributed to the presence of an ERE in the promoter region of the AQP3 gene in the ER$^+$ breast cancer cells. This observation was also in agreement with the present findings regarding AQP3 expression.

Immunocytochemical analysis showed that AQPs 1, 3, 4 and 5 exhibited distinct cellular localization. AQP1 was evenly distributed along the cytoplasm and surrounding the nucleus. AQP3 and AQP5 were localized predominantly in the nuclear region of all cell lines, except in pII cells, which showed some cytoplasmic staining for AQP5. In contrast, AQP4 was most strongly expressed in MCF10A cells, with low expression in breast cancer cell lines. The observed cellular distribution contrasts with published reports that have generally shown the AQPs to be located in the cytoplasm or cell membrane. For example, a study conducted by Luo *et al* (34)

demonstrated immunohistochemical localization of AQP1 in the cell membrane of breast cancer tissues, and at lower levels in the corresponding normal tissues. In their clinical study, they showed strong cytoplasmic AQP1 staining in the cells of invasive ductal carcinoma tissues and membranous staining in the cells of benign breast lesions (23). They further verified the cytoplasmic expression of AQP1 via *in vitro* analysis of primary breast cancer cells and MDA-MB-231 cells overexpressing AQP1. Shi *et al* (16) also detected the expression of AQPs 1, 4 and 5 in breast cancer and normal breast tissues, and found that AQPs 1 and 5 were located in the cellular membranes, whereas AQP4 exhibited membranous and cytoplasmic localization. Huang *et al* (26) showed that AQP3 exhibited cytoplasmic and membranous expression in the tumor cells of ER$^+$ breast cancer tissues. A recent study also investigated the cellular localization of AQPs 3 and 5 in triple negative breast cancer tissue, and found that both were expressed in the membrane and the cytoplasm (35). In

view of these reports, the immunolocalization experiments were repeated to detect AQP3 in pII cells with antibodies from several commercial sources, including those used in the abovementioned studies, as well as using different fixation methods to determine whether this may account for the disparity of these observations. The results obtained with antibodies from 3 different companies all consistently confirmed the nuclear localization of AQP3 in pII cells. AQP1 was the only AQP to exhibit consistent cytoplasmic localization; AQP4 and AQP5 showed some cytoplasmic and membranous distribution in certain cell lines. Western blot analysis using the same anti-AQP3 antibody identified a band of the expected molecular weight of 32 kDa, which supports the specificity of these antibodies.

Only the ER-silenced pII cells are affected by alkaline pH and exhibit blebbing. Neither normal breast cells nor ER$^+$ cells show this phenomenon. ER$^-$ MDA-MB-231 cells do undergo some blebbing, but not at comparable levels to pII cells (13,14,36). Therefore, the pH experiments were only conducted using pII cells. At present, no other stimuli that cause blebbing without inducing apoptosis have been identified. Notably, exposure to acidic pH does not induce this response (14). In the current study, the alterations in the morphology of pII cells exposed to alkalinity were further verified. As the formation of the blebs involves dynamic cytoplasmic streaming, this would presumably require substantial water flux, which is generally hypothesized to be controlled by the AQPs to maintain osmolality. Thus, the effects of changing the medium of pII cells that had previously been exposed to alkaline pH on bleb formation were examined, with the medium replaced with culture medium diluted with various ratios of water or sucrose. As would be expected the cells enlarged with increasing hypotonicity of the medium, and the blebs disappeared. Notably, the cells did not burst even after 90-120 min of observation. The redistribution of some AQP subtypes into the cellular blebs may aid to maintain the integrity of the cell and support the cytoskeleton. Under hypertonic conditions, there was also a reduction in the blebbing. As these were only exploratory observations, it will be interesting to examine the expression and distribution of AQPs during this process in further experiments. Indeed, Monzani *et al* (37) demonstrated that downregulation of AQP1 in a human melanoma cell line and a human microvascular cell line altered the organization of F-actin. In addition, AQP1 was found to co-immunoprecipitate with Lin-7 in these cell lines; a model was proposed that AQP1 acts as a scaffolding protein of the plasma membrane that is involved in the organization of the cytoskeleton by interacting with the Lin-7/β-catenin pathway (37). In the present study, only AQP1 and AQP3 were observed to be translocated into the formed blebs. The redistribution of these proteins into the blebs suggests their involvement in the process of bleb formation and/or stabilization and, by implication, in cell migration and invasion. The process of cell migration has been associated with membrane protrusions such as lamellipodia and ruffling at the leading edge of migrating cells (38). Hu and Verkman (39) reported that AQP1 showed polarized distribution at the leading edge of migrating mouse melanoma B16F10 cells and was associated with increased migration. In the present study, siRNA-mediated knockdown of AQP3 in pII cells resulted in a significant reduction in cell migration

and invasion through BME; the percentage of blebbing cells was also significantly reduced upon subsequent exposure to alkaline pH. It should be noted that a proportion of the transfected cells still blebbed despite loss of AQP3. This is not unexpected, as our previous studies showed that several other molecules and signaling pathways are involved in the complex process of blebbing in pII cells (13,14,40); these would be unaffected by AQP3 knockdown.

A number of studies have suggested that AQP3 is associated with cancer progression and metastasis in esophageal, colorectal, hepatocellular and gastric cancers (21,41-43). Hara-Chikuma and Verkman (33) demonstrated that AQP3 contributes to breast cancer cell migration by facilitating the transport of hydrogen peroxide. Another study showed that lentiviral shRNA-mediated knockdown of AQP3 in MDA-MB-231 and Bcap-37 breast cancer cell lines significantly reduced fibroblast growth factor (FGF)-2-induced cell migration (28). In addition, FGF receptor (FGFR) kinase, PI3K and mitogen-activated protein kinase kinase 1/2 inhibitors reduced FGF-2-dependent increases in AQP3 expression and cell migration, indicating the involvement of the FGFR-PI3K and FGFR-ERK signaling pathways. Another study reported that siRNA-mediated knockdown of AQP3 in the ER$^+$ breast cancer cell lines T47D and MCF7 led to a prominent reduction in cell migration; upregulation of this protein in T47D cells markedly increased both processes (26). It was found that AQP3 regulates estradiol-induced breast cancer cell migration and invasion by inducing reorganization of the actin cytoskeleton (including formation of filopodia) and affecting the expression of EMT inducer molecules, favoring the metastasis of breast cancer (33). Similarly, transfection of AQP3 shRNA into MDA-MB-231 breast cancer cells reduced cell proliferation, migration and invasion (44). However, downregulation of AQP3 was also associated with a marked decrease in the viability of cells exposed to 100 μM 5-fluorouracil, a fluoropyrimidine pro-drug administered to patients with breast or colorectal tumors (44).

It should be considered that the process of cancer cells attaining motility and the capacity for invasion is a complex process involving numerous molecular participants, and evaluating only one of these molecules at a time ignores the contributory effect of others; this has been demonstrated in our previous studies by the wide range of agents that inhibit blebbing (13,14,36). Interpretation of data should take this into consideration. A shortcoming of siRNA knockdown is the incomplete silencing of the gene of interest, which may be more effectively achieved using gene editing techniques such as CRISPR. Further direct evidence is also required to directly connect the AQPs with the osmotic effects on blebbing.

In conclusion, the expression of AQPs 1, 3, 4 and 5 is modulated in breast cancer; all of them were upregulated in breast cancer cells compared with normal breast cells, except for AQP4, which was more intensely expressed in normal breast cells. AQP3 serves a significant role in endocrine-resistant breast cancer cell motility and invasion, and may participate in the processes leading to the blebbing of endocrine-resistant cells. Therefore, AQP3 (and potentially other AQPs) may represent additional therapeutic targets in the prevention of metastatic spread of breast cancers.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MAK and YAL conceived the study and designed the experiments. AEA with some assistance from SK performed the experiments. AEA, MAK and YAL analyzed the data. YAL and MAK contributed reagents, materials and analytical tools. MAK, AEA and YAL drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

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Competing interests

The authors declare that they have no competing interests.

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